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# Severity of Human African Trypanosomiasis in East Africa Is Associated with Geographic Location, Parasite Genotype, and Host Inflammatory Cytokine Response Profile

Lorna MacLean,[1] John E. Chisi,[2] Martin Odiit,[3] Wendy C. Gibson,[4] Vanessa Ferris,[4] Kim Picozzi,[5] and Jeremy M. Sternberg[1]*

*College of Medicine, University of Malawi, Blantyre, Malawi[2]; Sleeping Sickness Special Programme, LIRI, Tororo, Uganda[3]; and School of Biological Sciences, University of Bristol, Bristol,[4] CTVM, University of Edinburgh, Easter Bush, Roslin,[5] and School of Biological Sciences, University of Aberdeen, Aberdeen,[1] United Kingdom*

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**The mechanisms underlying virulence in human African trypanosomiasis are poorly understood, although studies with experimental mice suggest that unregulated host inflammatory responses are associated with disease severity. We identified two trypanosomiasis foci with dramatically different disease virulence profiles. In Uganda, infections followed an acute profile with rapid progression to the late stage (meningoencephalitic infection) in the majority of patients (86.8%). In contrast, infections in Malawi were of a chronic nature, in which few patients progressed to the late stage (7.1%), despite infections of several months' duration. All infections were confirmed to be *Trypanosoma brucei rhodesiense* by testing for the presence of the serum resistance-associated (*SRA*) gene, but trypanosomes isolated from patients in Uganda or Malawi were distinguished by an *SRA* gene polymorphism. The two disease profiles were associated with markedly different levels of tumor necrosis factor alpha (TNF-α) and transforming growth factor β (TGF-β) in plasma. In Uganda but not Malawi early-stage TNF-α was elevated, while in Malawi but not Uganda early-stage TGF-β was elevated. Thus, rapid disease progression in Uganda is associated with TNF-α-mediated inflammatory pathology, whereas in the milder disease observed in Malawi this may be ameliorated by counterinflammatory cytokines. These differing host responses may result either from differing virulence phenotypes of northern and southern trypanosomes or from immune response polymorphisms in the different host populations.**

Human African trypanosomiasis (HAT), or sleeping sickness, is transmitted by tsetse flies in sub-Saharan Africa. Following inoculation of infective organisms, the parasites initially proliferate at the site of a fly bite, causing local inflammation (chancre), and subsequently invade the hemolymphatic system. This is known as the early stage of the disease. Later in infection the parasites invade the central nervous system, causing the meningoencephalitic, or late, stage of the disease. Without treatment, the disease progresses to coma and death. Infection models in mice indicate that African trypanosomes trigger potent inflammatory responses, and it has been suggested that survival is determined by the ability of different inbred strains to regulate inflammatory pathology (19). So far, it has been difficult to reconcile these results with the limited number of studies of cytokine responses in HAT patients, although high levels of both tumor necrosis alpha (TNF-α) and interleukin 10 (IL-10) have been associated with infection (15, 16)

HAT is caused by two subspecies of *Trypanosoma brucei*: *T. b. rhodesiense* is found in East Africa, and *T. b. gambiense* is found in West and Central Africa. HAT caused by *T. b. rhodesiense* is more acute and progresses more rapidly to the late stage than *T. b. gambiense* infection. The two subspecies can be distinguished genetically, and in particular the human serum resistance-associated (*SRA*) gene defines *T. b. rhodesiense* (10, 27) and plays a functional role in permitting human infectivity (23). While *T. b. rhodesiense* infections in humans are typically described as being acute, there is a spectrum of disease severity and speed of progression to central nervous system infection. There is a longstanding belief that the disease is chronic in southern countries of East Africa, with human carriers having been reported, and increases in virulence towards the north (20) and genetic characterization of *T. b. rhodesiense* isolates have shown differences between northern and southern isolates (9, 12, 13). More recently, it has been shown that northern and southern isolates differ in allelic polymorphisms in the *SRA* gene and its genomic environment (10, 11). Despite these differences, to the best of our knowledge no comparative studies of HAT in the northern and southern foci of East Africa have been carried out.

To study the hypotheses that there are two different types of HAT caused by *T. b. rhodesiense* in East Africa and that increasing disease virulence is correlated with inflammatory response, we have systematically compared disease progression, host cytokine response, and parasite genotype in patients recruited in Uganda (northern type) and Malawi (southern type).

* Corresponding author. Mailing address: School of Biological Sciences, University of Aberdeen, Zoology Building, Aberdeen AB24 2TZ, United Kingdom. Phone: 01224 272272. Fax: 01224 272396. E-mail: j.sternberg@abdn.ac.uk.

## MATERIALS AND METHODS

**Study sites and subjects.** Twenty-eight HAT patients and sixteen noninfected control individuals were recruited in Nkhotakota, central Malawi, in 2002. Of these, 12 were identified with signs of HAT during community surveillance, and

the remainder presented directly at Nkhotakota District Hospital. Ninety-one HAT patients and seventy-one noninfected control individuals were recruited in Soroti and Tororo districts in eastern Uganda between 1998 and 2000. Ten patients were identified during community surveillance, and the remainder presented directly to local hospitals. We have previously presented plasma cytokine levels from the Ugandan patients but not controls (16). Diagnosis of HAT was by microscopic detection of trypanosomes in wet blood films, Giemsa-stained thick blood films, or the buffy coat fraction after microhematocrit centrifugation (26). Following admission, an extensive clinical history was taken from each patient. Stage determination was by microscopic examination of cerebrospinal fluid (CSF) after lumbar puncture (3). Patients with no trypanosomes in the CSF and white blood cell counts of $\leq 5$ cells/mm$^3$ were classified as having early-stage disease, while patients with trypanosomes in the CSF and/or white blood cell counts of $>5$ cells/mm$^3$ were classified as having late-stage disease. Early-stage infection was treated with suramin, and late-stage infection was treated with melarsoprol (16). Subjects or their guardians signed consent forms after receiving standard information in their local language. Protocols were approved by ethics committees in Aberdeen, Uganda (Ministry of Health), and Malawi (College of Medicine). Malaria-parasitemic and microfilaremic individuals were excluded from the study.

Blood samples taken before treatment commenced were collected in EDTA Vacutainers (Greiner, Stroud, United Kingdom) and centrifuged for 10 min at $3{,}000 \times g$. Platelet-depleted plasma was aliquoted and frozen immediately in liquid nitrogen. Trypanosome DNA was sampled by applying a 200-μl suspension taken from the buffy coat layer to FTA cards (Whatman Bioscience, Maidstone, United Kingdom), which were dried and stored at room temperature.

**Cytokine assays.** Gamma interferon (IFN-γ), IL-10, and transforming growth factor β (TGF-β) concentrations were measured using a solid-phase sandwich enzyme-linked immunosorbent assay (OptiEIA; BD Pharmingen, Cowley, United Kingdom). Biologically active TNF-α was measured using the BioLISA system (Bender MedSystems, Vienna, Austria), in which TNF receptor bound to the solid phase acted as the capture ligand.

**PCR amplification and sequencing.** A 2-mm-diameter punch was taken from each fluorescent treponemal antibody card and processed according to the manufacturer's instructions. These were then used as template for each PCR. In addition, blood samples from three noninfected individuals were used as negative controls, and trypanosomes of *T. b. brucei* STIB247 were used as a negative control for the *SRA* PCR.

Two sets of primers were used to screen for *T. brucei*: Orphon 5J-u and 5J-l (4) and Museq 1 and 2 (14). The final $MgCl_2$, deoxynucleoside triphosphate, and primer concentrations were 2.5, 0.2, and 1 μM, respectively, in a 50-μl reaction mixture with 2 U of Immolase DNA polymerase (Bioline, London, United Kingdom). The standard amplification program was one 7-min cycle at 95°C followed by 40 cycles of 60 s at 95°C, 60 s at 60°C, and 60 s at 72°C. To identify *T. b. rhodesiense*, primers to the *SRA* gene, which is only present in *T. b. rhodesiense*, were designed. There is considerable sequence homology between the *SRA* gene and members of the *T. brucei* variant surface glycoprotein gene family; however, there is a 378-bp deletion in the *SRA* gene which encodes part of the variant surface glycoprotein gene N-terminal domain. Primers SRA F1 (5′-AGT GCC TGT ATC GCC CA) and SRA R1 (5′-CTT GGA TTG ATA CGA GTT TGC) were designed with reference to the published Ugandan *SRA* gene sequence (GenBank accession no. Z37159) to amplify across this deletion, giving a 772-bp product if *SRA*-positive or a 1,150-bp product if *SRA*-negative trypanosomes are present. The amplification conditions were the same as the standard conditions above but used an annealing temperature of 57°C and one further extension cycle of 5 min at 72°C.

Comparison of *SRA* genes from northern and southern isolates of *T. b. rhodesiense* (GenBank accession no. AJ345057 and AJ345058) previously revealed a region of high polymorphism at the 3′ end of the gene (10). Primers *SRA*-M (5′-CTG CTT CTG CTT TTT CC) and *SRA*-K (5′-TTT CTG CGG CTT TTG TG) were designed to exploit this difference in order to differentiate between the northern and southern alleles of the *SRA* gene, respectively. When used in conjunction with a common primer (*SRA*-C, 5′-GGA AGC GCT ACT TTC AAT CG), primer *SRA*-M or *SRA*-K amplifies a 443-bp product. PCR conditions for *SRA*-C–*SRA*-M and *SRA*-C–*SRA*-K were the same as the standard conditions above but used 45 cycles and an annealing temperature of 62°C. Two Malawian *SRA*-C–*SRA*-K and two Ugandan *SRA*-C–*SRA*-M PCR products were cloned into pGem T Easy (Promega, Southampton, United Kingdom) and sequenced using M13 primers.

**Statistical analyses.** The Mann-Whitney U test was used for all analyses unless otherwise stated.

TABLE 1. Clinical characteristics of study groups

| Subject group | No. of subjects (male:female) | Median age (yr) (range) | No. of subjects in late stage (% of total) | No. of subjects with chancre (% of total) | Median duration of disease (days) (interquartile range)[a] |
|---|---|---|---|---|---|
| Malawi | 15:13 | 23 (2–55) | 2 (7.1) | 0 (0) | 30 (142) |
| Uganda | 59:32 | 29 (2–80) | 79 (86.8) | 26 (28.6) | 21 (24) |

[a] As reported by early-stage patients.

## RESULTS

**HAT characteristics in Uganda and Malawi.** A total of 86.8% of HAT cases in Uganda were classified as late stage on admission, while only 7.1% of HAT cases in Malawi had progressed to this stage (Table 1). Patient histories revealed that of those in the early stage on admission, the median duration of illness in Malawian patients was significantly longer (30 days) than that in Ugandan patients (21 days; $P < 0.05$). Among early-stage patients, 9 patients out of 28 (32.1%) in Malawi but only 1 patient out of 12 (8.3%) in Uganda reported illness longer than 90 days. Of these, 6 patients (21%) in Malawi remained in the early stage after reporting an illness of 180 days. None of the patients recruited in Malawi exhibited a chancre, whereas 28.6% (58.3% of early- and 24.7% of late-stage) of Ugandan patients had a chancre. As the chancre is one of the earliest-presenting clinical signs in HAT, typically appearing within 5 to 15 days at the site of tsetse bite and persisting for 2 to 3 weeks (8), this implies that many Ugandan patients had been infected quite recently.

**Disease progression and host inflammatory and counterinflammatory responses.** To determine if disease progression is associated with the inflammatory and counterinflammatory cytokine milieus, patient and control plasma cytokine levels were analyzed. As only two late-stage cases were identified in Malawi, the analysis undertaken includes early- and late-stage Ugandan patients but only early-stage Malawian patients. In all HAT cases, IFN-γ and IL-10 (Fig. 1a and b) were significantly elevated at levels similar to those described previously (16); however, there were significant differences in the profiles of TGF-β and TNF-α between Uganda and Malawi. In early-stage Ugandan patients, plasma TNF-α was significantly elevated (median, 70.7 pg/ml) over both local control levels (median, 32.9 pg/ml; $P < 0.05$) and Malawian patient levels (median, 11.0 pg/ml; $P < 0.05$). In late-stage Ugandan patients and Malawian patients, plasma TNF-α was not significantly elevated over local control levels. There was no significant increase in TGF-β levels in early-stage Ugandan patients, but the plasma concentration of this cytokine was significantly increased over local control levels in Malawian patients (median, 8,396 versus 351 pg/ml; $P < 0.001$) and late-stage Ugandan patients (median, 4,480 versus 283 pg/ml; $P < 0.001$). Also, when compared directly, TGF-β levels in Malawian early-stage patients were significantly higher than those in Ugandan early-stage patients (median, 8,396 versus 398 pg/ml; $P < 0.001$).

There was no significant association between plasma cytokine levels and patient age or sex in either study location.

**Parasite *SRA* genotype in Uganda and Malawi.** Parasite DNA was isolated from 18 Malawian and 3 Ugandan patients.

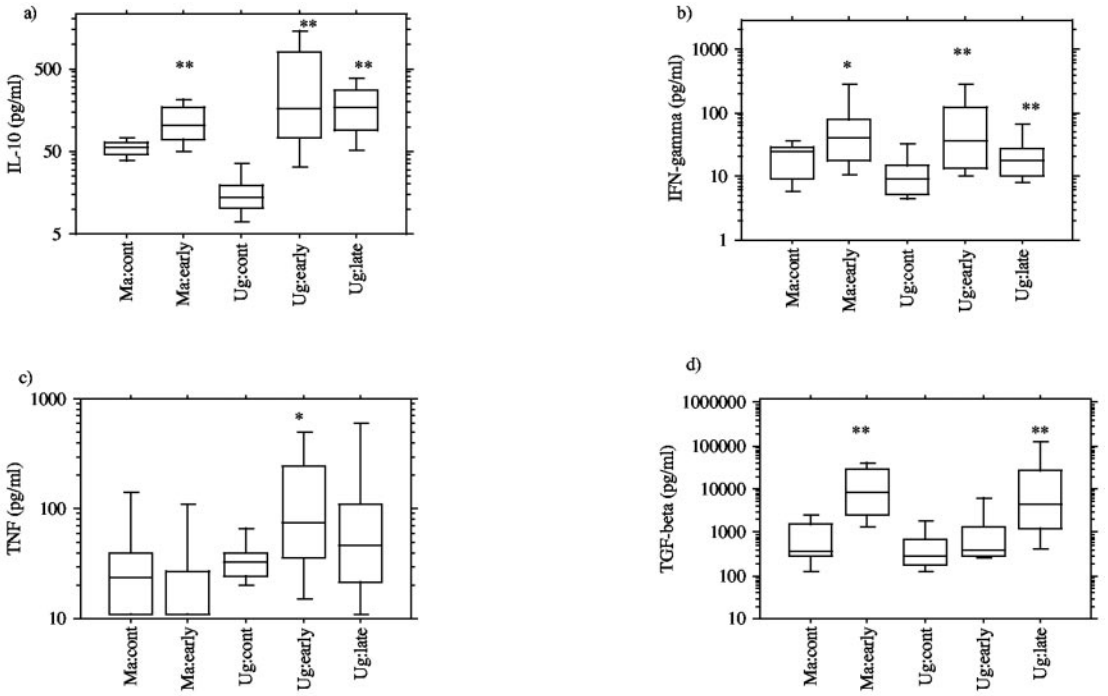


FIG. 1. Plasma IL-10 (a), IFN-γ (b), TNF-α (c), and TGF-β (d) concentrations in trypanosomiasis patients. Boxes indicate medians and interquartile ranges, and whiskers represent 10th and 90th percentiles. Asterisks indicate significant increases over the local control group: **, $P < 0.001$; *, $P < 0.05$ (Mann-Whitney U test). Group abbreviations: Ma, Malawi ($n = 26$); Ug, Uganda ($n = 91$); cont, control (Malawi, $n = 16$; Uganda, $n = 71$).

A 246- or 286-bp PCR product was produced with primer pair Orphon 5J-l and 5J-u or Museq 1 and 2, respectively (Fig. 2), confirming that the samples were *T. brucei* spp., and we then determined if the parasites were *T. b. rhodesiense* by PCR amplification of the *SRA* gene (Fig. 2). DNA from all trypanosome isolates yielded a 772-bp product by PCR with *SRA*-F1 and R1 primers, confirming the parasites to be *T. b. rhodesiense*. A control using DNA from *T. b. brucei* STIB247 yielded only a 1,150-bp product and therefore was *SRA* negative.

Previous analysis has shown that two sequence variants of the *SRA* gene exist: the northern variant was found in all *T. b. rhodesiense* isolates from Uganda, Kenya, and Tanzania, while the southern variant was found only in isolates from Zambia and Ethiopia (9). Here, PCR primers were designed to distinguish the two variants without recourse to DNA sequence

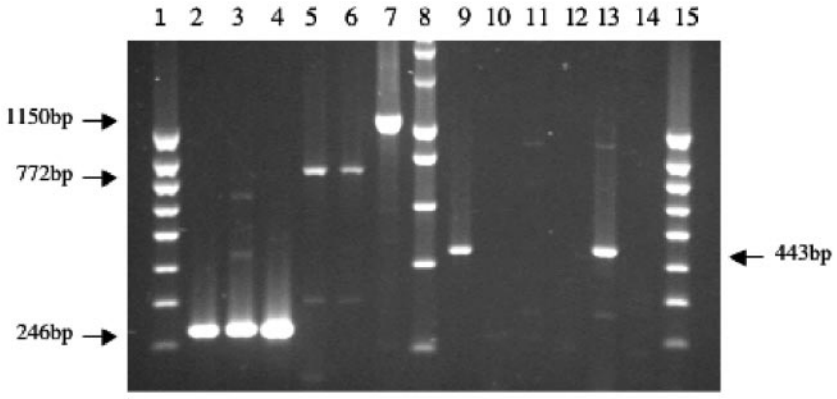


FIG. 2. PCR amplification of *T. brucei sp.* 246-bp product with Orphon 5J-u and 5J-l primers is shown for Malawian isolate NKK/T/10 (representative of results for 18 different patient isolates), Ugandan isolate SER005 (representative of results for 3 different patient isolates), and *T. b. brucei* 247 (lanes 2, 3, and 4, respectively). Amplification of the SRA gene 772-bp product with SRA F1/R1 primers is shown for isolates NKK/T/10 and SER005 (lanes 5 and 6), but a 1,150-bp product is shown for *T. b. brucei* 247 (lane 7); thus, no SRA gene is present. SRA gene southern-type-specific polymorphism C/K amplified a 443-bp product with NKK/T/10 (lane 9) but not SER005 or *T. b. brucei* 247 (lanes 10 and 11). Northern-type-specific C/M primers amplified a 443-bp product with SER005 (lane 13) but not NKK/T/10 or *T. b. brucei* 247 (lanes 12 and 14). Markers are Bioline Hyperladder IV (lanes 1 and 15) and I (lane 8).

analysis; the specificity of these primer combinations for *SRA* alleles of the northern or southern type was verified using a range of *T. b. rhodesiense* isolates of known *SRA* type (10) and sequence analysis of selected PCR products. As expected, the *SRA* allele of the Ugandan isolates was of the northern type, giving a 443-bp product with the northern-type-specific *SRA*-C–*SRA*-M primer pair, but not the southern-type-specific *SRA*-C–*SRA*-K primer pair (Fig. 2). In contrast, all the Malawian isolates had *SRA* alleles of the southern type. The *SRA* gene nucleotide sequence from two of the Malawian isolates spanning the *SRA*-B537 (25) and *SRA*-K primers was identical to the previously reported *SRA* gene from Zambian isolate 058 (10) (GenBank accession no. AJ345058, nucleotides 125 to 1018). Two Ugandan *SRA*-B537 and *SRA*-M primer PCR products were also sequenced. They were identical and showed 100% homology to the previously reported northern-type *SRA* sequence (GenBank accession no. AJ345057).

## DISCUSSION

This is the first systematic study comparing *T. b. rhodesiense* virulence between northern (Ugandan) and southern (Malawian) disease foci, although the first reports of a milder form of the disease in southern countries was published more than 60 years ago (20). The results provide evidence of two types of disease, each associated with distinct host inflammatory cytokine profiles and parasite *SRA* gene variants. One of the early-presenting clinical signs is the development of a chancre at the site of a tsetse bite within 5 to 15 days (8). This lesion subsides after 2 to 3 weeks and, therefore, can be used as an indication of the length of time an individual has been infected with trypanosomes. In this study none of the Malawian HAT patients presented with a chancre, although 92.8% were classified as having early-stage disease, suggesting that either these patients had been infected for longer than 4 weeks before diagnosis or the infecting trypanosome did not cause the development of a chancre. In contrast, the overall incidence of chancre in Ugandan patients was 58.3% in early- and 24.7% in late-stage cases. This implies that most early-stage patients had been infected for no longer than 4 to 5 weeks and that in this time a quarter of the patients had progressed to the late stage. We also questioned patients on the duration of illness before diagnosis. While such data are imprecise due to differing patient perceptions of disease, the results are completely consistent with the chancre data and support the view that Malawian patients had been infected for considerably longer on average than Ugandan patients. Given the apparent duration of infection in the Malawian patients, most were expected to have progressed to the late stage. Instead, 92.8% of Malawian patients had early-stage infections on admission, indicating a more chronic disease profile in Malawi. In comparison, although Ugandan patients had been infected for a shorter period, 86.8% had late-stage infections, again demonstrating an acute disease course.

As the chronic presentation of HAT in Malawi shared some features of *T. b. gambiense* infection, where absence of chancre is less frequent (5), we undertook molecular genotyping of trypanosomes from Uganda and Malawi. The *SRA* gene, which is present in *T. b. rhodesiense* but not *T. b. gambiense* (9, 22), was used to confirm the identity of the trypanosome. All trypanosomes isolated from HAT patients in both Uganda and Malawi had the *SRA* gene and were thus confirmed as *T. b. rhodesiense*.

Despite the high degree of sequence homology (97.9 to 99.7%) in the *SRA* gene across a wide geographical range of *T. b. rhodesiense* isolates (10), sequence differences allow northern and southern variants to be distinguished. In this study we found that Malawian and Ugandan trypanosomes were of the southern and northern *SRA* genotypes, respectively. Previous isoenzyme and restriction fragment length polymorphism strain characterization studies have revealed that *T. b. rhodesiense* isolates fall into northern and southern groups (12, 13), and the *SRA* gene appears to be a marker for these two genotypes (9).

Studies of trypanosomiasis in mouse models have suggested that the balance between pro- and counterinflammatory cytokines plays a significant role in determining the production of a protective or disease-exacerbating host immune response (19). The data presented here corroborate this hypothesis for the first time with respect to HAT. Although IL-10 and IFN-γ were elevated over control levels in all patients, when TNF-α and TGF-β were measured, marked differences between the two study areas and the stage of disease were apparent. In Ugandan early-stage patients, plasma concentrations of TNF-α were significantly increased, while in Malawian patients, TGF-β concentrations were increased but TNF-α remained at control levels. In late-stage Ugandan patients, a significant increase in the level of TGF-β was paralleled by a reduction in the level of TNF-α. Whether these differences are directly related to the clinical stage of infection or the overall duration of infection cannot be resolved with our data, as it is possible that the lower TNF-α levels in Malawian patients reflect a more extended period of infection prior to diagnosis. However, there is a clear association between TNF-α levels and disease severity. Studies using mouse models have indicated that TNF-α is associated with immune dysfunction (17) and neuropathogenesis (21), and it has also been proposed that inflammatory mediators, such as TNF-α, play a role in blood-brain-barrier dysfunction, enabling entry of trypanosomes into the central nervous system and thus initiating the late stage of infection (6). The high level of plasma TNF-α could therefore be involved in the apparent rapid progression to late-stage disease observed in Uganda, while the high levels of TGF-β in the plasma of the Malawi patients and in the late-stage Uganda patients may result in the down-regulation of TNF-α, ameliorating pathology and allowing prolonged survival. Although TGF-β has both pro- and anti-inflammatory roles depending on its environment and concentration (24), at high concentrations it plays an anti-inflammatory role by suppressing macrophage secretion of TNF-α and nitric oxide (7) and IFN-γ and TNF-α production in NK cells (1).

Understanding the fundamental basis of the distinct immunological responses and disease patterns in Malawi and Uganda is now of prime importance, and we propose that two hypotheses provide a framework for future work. In the first, the southern and northern parasite genotypes may have divergent virulence phenotypes. While northern-southern polymorphism in the *SRA* gene differentiating the Malawian and Ugandan parasites is in itself unlikely to have any direct phenotypic effect on disease progression and immune response, there are

likely to be polymorphisms at other loci encoding putative virulence factors. These may be totally novel molecules or trypanosome products which have previously been shown to interact with and modulate macrophage inflammatory responses in experimental models (18). The alternative hypothesis attributes the cause of the difference in disease in Malawi and Uganda to differences in genetic resistance to trypanosomiasis among host populations. This is an idea which was first put forward over 25 years ago as a result of studies of a mild form of HAT in Zambia (2). It was proposed that peoples of Bantu descent, whose ancestors are likely to have been exposed to human infective trypanosomes for several thousand years, may have greater tolerance of trypanosome infection that those of Nilotic descent, who migrated into the East African region from tsetse-free areas to the North over the past 2,000 years. This hypothesis is consistent with our data, since the population of Malawi is largely of Bantu ancestry, while a significant population in eastern Uganda are the Ateso, who are of Nilotic ancestry.

Resolution of these two hypotheses will provide a major advance in our understanding of the pathogenesis of trypanosomiasis and offer opportunities for novel immunological interventions to manage the disease.

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